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Prophage Excision Activates *Listeria* Competence Genes that Promote Phagosomal Escape and Virulence

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SUMMARY

The DNA uptake competence (Com) system of the intracellular bacterial pathogen *Listeria monocytogenes* is considered nonfunctional. There are no known conditions for DNA transformation, and the Com master activator gene, *comK*, is interrupted by a temperate prophage. Here, we show that the *L. monocytogenes* Com system is required during infection to promote bacterial escape from macrophage phagosomes in a manner that is independent of DNA uptake. Further, we find that regulation of the Com system relies on the formation of a functional *comK* gene via prophage excision. Prophage excision is specifically induced during intracellular growth, primarily within phagosomes, yet, in contrast to classic prophage induction, progeny virions are not produced. This study presents the characterization of an active prophage that serves as a genetic switch to modulate the virulence of its bacterial host in the course of infection.

INTRODUCTION

Listeria monocytogenes is a Gram-positive facultative intracellular pathogen that invades a wide array of mammalian cells. Upon invasion, *L. monocytogenes* initially resides in a membrane-bound compartment from which it must escape into the host cell cytosol (Hamon et al., 2006). In the cytosol, the bacteria replicate and use the host actin polymerization machinery to propel themselves on actin filaments within the cell and from cell to cell (Tilney and Portnoy, 1989). Escape from the membrane-bound compartment (vacuole) is a critical step in *L. monocytogenes* pathogenesis, because failure to reach the cytosol results in avirulent infection. Although *L. monocytogenes* is capable of replicating within specialized vacuoles (Birmingham et al., 2008), a failure to escape matured phagosomes generally leads to bacterial degradation and killing (Herskovits et al., 2007).

L. monocytogenes encodes several virulence factors that are required for its escape from the initial and secondary vacuoles during cell-to-cell spread. Lysis of the vacuole is largely mediated by the pore-forming hemolysin, Listeriolysin O (LLO), encoded by the *hly* gene (Cossart et al., 1989; Kathariou et al., 1987; Portnoy et al., 1988). Together with LLO, *L. monocytogenes* secretes two phospholipases, phosphoinositol-PLC (PlcA) and phosphatidylcholine-PLC (PlcB), that facilitate the escape of the bacteria from the vacuole (Smith et al., 1995). Although extensive research has focused on *L. monocytogenes* vacuolar escape, the exact mechanism underlying this critical step remains unclear.

The competence (Com) system is known to facilitate exogenous DNA uptake across bacterial membranes by a process termed DNA transformation (Dubnau, 1999). DNA transformation plays an important role in inter- and intraspecies gene transfer and in DNA repair (Claverys et al., 2006). Bacteria that undergo natural DNA transformation are considered competent, in what is referred to as a controlled physiological state. The Com system of Gram-positive bacteria has been studied at length in *Bacillus subtilis* and shown to be regulated by a peptide-pheromone sensing mechanism. In brief, a small peptide pheromone is exported outside the bacteria, where it is sensed by a two-component system that in turn activates a series of events that ultimately stabilize the Com master transcriptional activator, ComK. Subsequently, ComK induces expression of the late *com* genes, which are responsible for the assembly of the Com apparatus (Claverys et al., 2006).

The late *com* genes are clustered in three separate operons: the *comG* operon, the *comE* operon, and the *comF* operon. The *comG* operon encodes several prepilin proteins that are assembled into a pseudopilus that crosses the cell wall as well as two additional proteins required for its biogenesis: ComGA, a traffic ATPase that is associated peripherally with the inner side of the cell membrane, and ComGB, an integral membrane protein. The *comE* operon encodes ComEA, which functions as a DNA receptor that binds DNA extracellularly; ComEB, which has a unknown function; and ComEC, which is a polytopic membrane protein that forms the membrane translocation channel. The *comF* operon encodes ComFA, which is an intracellular DNA-helicase required for DNA transport, and ComFB and ComFC, which have unknown functions. The *Listeria* genomes contain most of the late *com* gene homologs (except for *comFB*); however, all of the regulatory genes that have been characterized in *B. subtilis* and *Streptococcus pneumoniae*

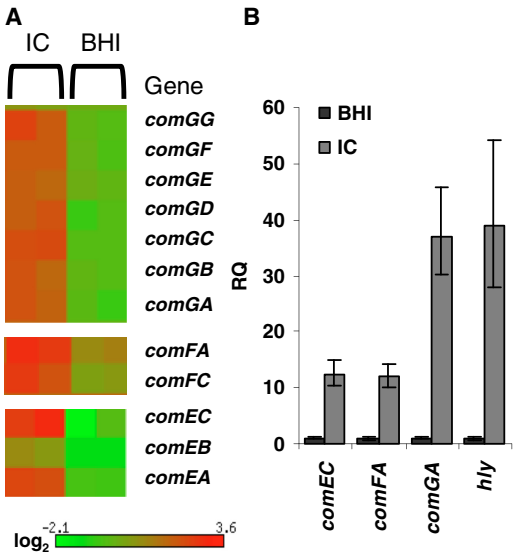

Figure 1. *L. monocytogenes* Competence Genes Are Induced during Intracellular Growth

(A) Microarray analysis of gene expression in *L. monocytogenes* grown intracellularly in macrophage cells for 6 hr relative to gene expression in bacteria grown in BHI medium to mid-exponential phase. The heat map represents two independent biological repeats.

(B) RT-qPCR analysis of late *com* genes transcription levels upon intracellular growth in macrophage cells for 6 hr and during mid-exponential growth in BHI medium. Transcription levels are represented as relative quantity (RQ), intracellular versus BHI medium growth. The data represent three biological repeats. Error bars represent the 95% confidence level.

are missing and no orthologs have been identified. The only remnant of the Com regulatory machinery in *Listeria*'s genome is the gene encoding for the major Com activator, ComK. Yet, this gene is interrupted in several *L. monocytogenes* strains by the insertion of a *Listeria*-specific prophage named A118 (Loessner et al., 2000). In *B. subtilis*, the expression of the late *com* genes and the ability to take up DNA are completely dependent on the activity of ComK (van Sinderen et al., 1995). To date, repeated attempts to transform *L. monocytogenes* have failed, even with strains containing the intact *comK* gene, suggesting that the role of the Com system has diverged in *Listeria* species (Borezee et al., 2000).

The temperate A118 prophage is specific to *L. monocytogenes* serovar 1/2 strains, which are associated with certain food-borne illness outbreaks. This bacteriophage belongs to the Siphoviridae family of double-stranded DNA bacterial viruses and has a long, noncontractile tail and an isometric head (Zink and Loessner, 1992). It was shown to adsorb to cell wall derivatives (Wendlinger et al., 1996) and to reproduce through both lysogenic and lytic cycles. In the lysogenic cycle, the phage's ~40-kb genome is integrated at a specific attachment site located within the *comK* gene, resulting in inactivation of this gene (Loessner et al., 2000). The phage attachment site comprises an unusual core sequence of only 3 nucleotides (GGA), which is conserved in the phage and the *comK* gene (Loessner et al., 2000). Upon UV irradiation, the phage enters the lytic cycle, producing progeny virions that are released via bacterial lysis. Bacterial lysis is accomplished by the combined action of phage-encoded holin and endolysin, which eventually perforate the bacterial membrane and digest its peptidoglycan (Loessner et al., 1995). Although the lifecycle of the A118 phage is well characterized, nothing is known about its impact on the behavior of *L. monocytogenes* during infection of mammalian cells.

An inspection of the genome sequence of *L. monocytogenes* 10403S strain revealed that a similar prophage to A118, named here ϕ10403S, is located within this strain's *comK* gene. Here we show that during intracellular replication of *L. monocytogenes* 10403S in macrophage cells, ϕ10403S-prophage is precisely excised, leaving an intact *comK* gene. The phage-free, intact *comK* gene produces a functional ComK protein that activates transcription of the Com system. The Com system is shown to be required for efficient phagosomal escape of *L. monocytogenes*, whereas Com components involved in DNA binding are dispensable. We describe a role for the Com system in *L. monocytogenes* and a unique regulatory mechanism that involves prophage excision.

RESULTS

The Late *com* Genes Are Transcriptionally Induced during *L. monocytogenes* Intracellular Growth

We noticed that the late *com* genes of *L. monocytogenes* are induced upon infection during a whole-genome transcriptome analysis of *L. monocytogenes* 10403S strain growing in bone marrow–derived (BMD) macrophage cells (L. Lobel and A.A.H., in press). As illustrated by microarray-based heat maps, all three operons of the late *com* genes (*comG*, *comF*, and *comE*) were specifically highly induced intracellularly, up to ~10-fold (Figure 1A). To validate the elevated intracellular expression levels of the *com* genes, we subjected representative genes from each *com* operon, as well as the virulence gene *hly* (encoding the LLO toxin), to real-time reverse transcription quantitative-PCR (RT-qPCR) analysis. Specifically, *comGA*, *comEC*, and *comFA* were each monitored at 6 hr postinfection (h.p.i.) of BMD macrophages. As shown in Figure 1B, all of the tested genes were highly induced intracellularly relative to their transcription levels during growth in a rich laboratory medium, brain heart infusion (BHI) broth. The transcriptional upregulation of *comGA* was particularly noticeable, comparable even to the transcriptional induction of *hly*, which is highly produced intracellularly.

The Competence Apparatus Promotes *L. monocytogenes* Virulence in a Manner Independent of DNA Uptake

The observed induction of *com* genes expression during infection prompted us to investigate whether these genes are necessary for *L. monocytogenes* intracellular growth. In-frame deletion mutants lacking either the whole *comG* (*comG*−) or whole *comE* (*comE*−) operons were generated, as well as single gene deletion mutants of various *com* genes (Table S1 available online). First, BMD macrophages were infected with *comG*−, *comE*−, or wild-type (WT) *L. monocytogenes*, and the ability of these bacteria to grow intracellularly was analyzed. Remarkably, we found that these mutants, which grew normally in BHI broth

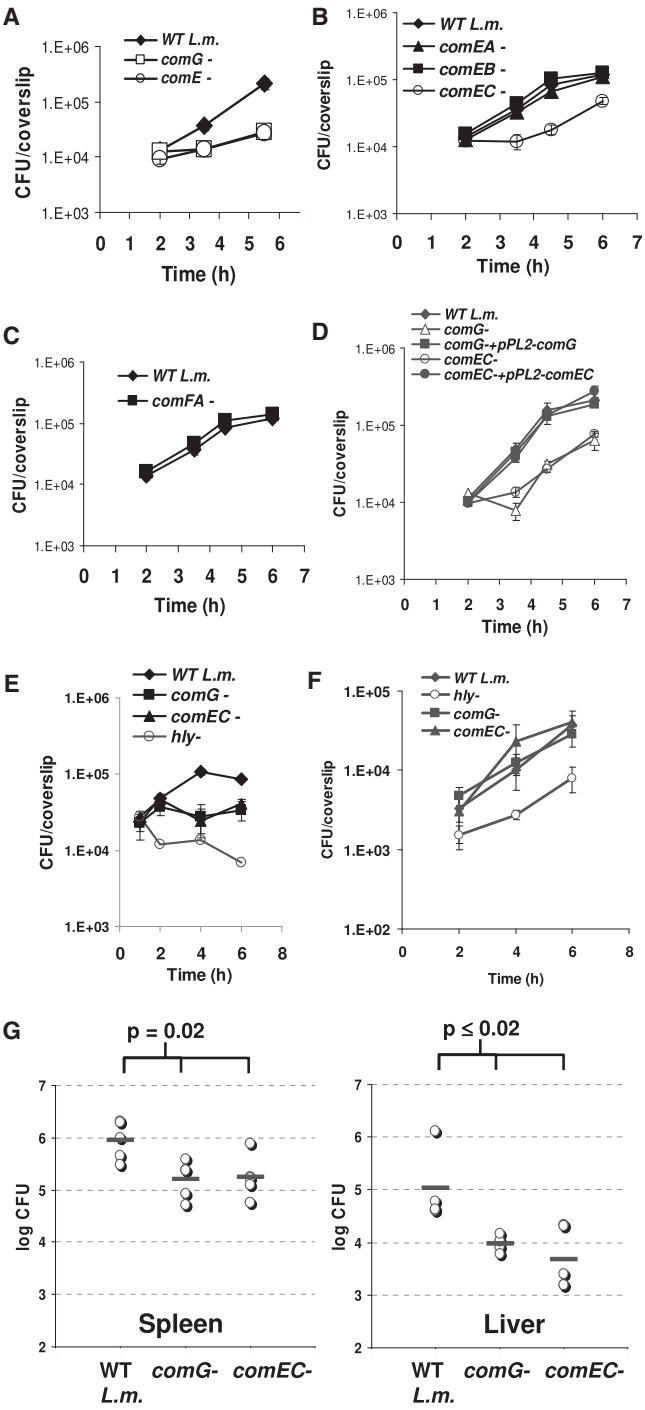

Figure 2. The Com Pseudopilus and Translocation Channel Are Required for *L. monocytogenes* Infection

(A) Intracellular growth curves of WT *L. monocytogenes* and *comG*− and *comE*− operon mutants grown in BMD macrophage cells.

(B) Intracellular growth curves of WT *L. monocytogenes* and *comEA*−, *comEB*−, and *comEC*− mutants grown in BMD macrophage cells.

(C) Intracellular growth curves of WT *L. monocytogenes* and the *comFA*− mutant grown in BMD macrophage cells.

(D) Intracellular growth curves of *comG*− and *comEC*− mutants and their complemented strains in BMD macrophage cells.

(E) Intracellular growth curves of WT *L. monocytogenes* and *comG*− and *comEC*− mutants grown in IFN-γ–activated BMD macrophage cells.

(F) Intracellular growth curves of WT *L. monocytogenes* and *hly*−, *comG*−, and *comEC*− mutants in HeLa cells.

(G) Intravenous infection of C57BL/6 mice with WT *L. monocytogenes*, *comG*− mutant, or *comEC*− mutant. Bacterial counts (CFUs) were numerated at 72 h.p.i. in the livers and spleens of five infected mice in each group. The p value was calculated using a t test. In all growth curves, the data represent three biological repeats. Error bars represent the SD.

See also Figure S1A and Table S1.

(Figure S1A), were severely defective in intracellular growth, as one log decrease in their number of colonies was observed at 6 h.p.i. (Figure 2A). As mentioned above, the *comG* operon encodes for proteins engaged in pseudopilus formation, effectively forming one functional unit, whereas the *comE* operon encodes for components with distinct functions. To assess directly whether the DNA binding/uptake function of the Com system is crucial for the ability of *L. monocytogenes* to grow intracellularly, we monitored the intracellular growth of bacteria lacking individual genes of the *comE* operon (*comEA*−, *comEB*−, and *comEC*−). Of the three mutants, we found that only the *comEC* gene (encoding the membrane translocation channel) was required for optimal intracellular growth, and the DNA binding receptor ComEA and ComEB proteins were dispensable (Figure 2B). Similarly, the ComFA DNA-helicase was also not necessary for intracellular growth (Figure 2C). The growth defects of the *comG*− and *comEC*− mutants were complemented by the *comG* operon and *comEC* gene, respectively, when these genes were introduced in *trans* with their native promoters using the integrative pPL2 plasmid (Lauer et al., 2002; Figure 2D; Table S1). These results establish that although most of the late *com* genes are induced intracellularly, only the cell-wall–crossing pseudopilus and the membrane translocation channel are required during infection of macrophage cells, indicating a role for the Com system in intracellular growth that is independent of DNA uptake.

To investigate the role of the ComG pseudopilus and ComEC channel during infection, we studied the ability of *comG*− and *comEC*− mutants to infect and grow within different cell types. First, we tested their adherence and invasion capabilities using the human epithelial Caco-2 cell line. The WT bacteria and *comG*− mutant were observed to adhere to and invade Caco-2 cells similarly (Figure S1B). Next, growth within IFN-γ-activated macrophages, which are less permissive for *L. monocytogenes* growth (Herskovits et al., 2007), was examined. As shown in Figure 2E, *hly*− mutant bacteria (deleted of LLO) are killed by these cells, whereas some WT bacteria succeed in escaping the phagosome and grow intracellularly. Of note, the number of colony-forming units (CFUs) of *comG*− and *comEC*− bacteria harvested from activated macrophages was constant throughout the 6 hr period of infection (Figure 2E). This observation raised the possibility that *comG*− and *comEC*− mutants are impaired in phagosomal escape, resulting in more bacteria becoming trapped and killed within the phagosomes. To explore this possibility further, we performed the converse experiment and examined the growth of *comG*− and *comEC*− mutants in HeLa cells, which are permissive for *L. monocytogenes* vacuolar escape (Gründling et al., 2003). Indeed, HeLa cells support the escape of

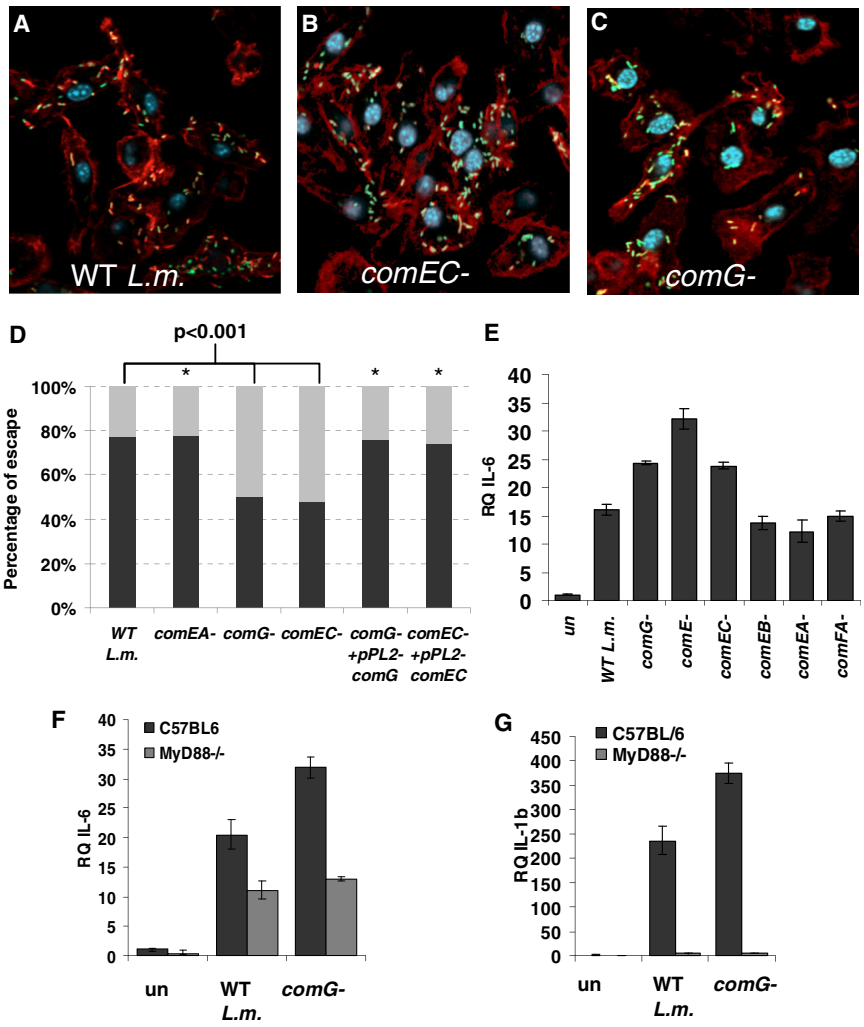

Figure 3. The Com Pseudopilus and the Translocation Channel Are Required for Efficient Phagosomal Escape

(A–C) Fluorescence confocal microscope images of BMD macrophages infected with (A) WT *L. monocytogenes*, (B) *comEC*− mutant, and (C) *comG*− mutant at 2.5 h.p.i. Bacteria are labeled with fluorescein-conjugated anti-*Listeria* antibody (green), macrophage nuclei with DAPI (blue), and actin with rhodamine phalloidin (red).

(D) Calculated percentage of bacterial escape. The results are representative of ten microscope images from two independent biological repeats for each strain; * indicates no significant difference compared with WT *L.m*.

(E) Induction of IL-6 cytokine transcription in BMD macrophages infected with WT *L. monocytogenes* or with indicated *com* mutants at 6 h.p.i.

(F) Induction of IL-6 cytokine and (G) IL-1β cytokine transcription in WT BMD macrophages and MyD88−/− deficient BMD macrophages infected with WT *L. monocytogenes* or the *comG*− mutant at 6 h.p.i. Transcription levels are presented as RQ relative to uninfected cells (un). The data represent three biological repeats. Error bars represent the 95% confidence level.

See also Figure S2.

The Com Apparatus Promotes *L. monocytogenes* Phagosomal Escape

To directly assess whether the *com* mutants are defective in phagosomal escape, we performed a phagosomal escape assay that is based on fluorescence microscopy (Glomski et al., 2002). This assay relies on the observation that bacteria within the cytosol nucleate host actin filaments on their surface, whereas bacteria in phagosomes do not. BMD macrophages were infected with select *com* mutants or WT bacteria and fixed and stained with rhodamine phalloidin, which binds host actin; DAPI, which stains nuclei; and fluorescein-conjugated anti-*Listeria* antibody. At 2.5 h.p.i., most of the WT bacteria (80%) were associated with actin tails within the macrophage cytosol (Figures 3A and 3D), whereas a large fraction of the *comG*− and *comEC*− mutants (50%) were labeled solely with fluorescein, indicating that they were still located within phagosomes (Figures 3B–3D). In control experiments, *comEA*− (the DNA receptor mutant that exhibited normal intracellular growth) escaped phagosomes in similarity to the WT bacteria, as well as the *comG*− and *comEC*− complemented strains (Figure 3D).

Additional evidence supporting the view that the *comG*− and *comEC*− mutants are indeed delayed within phagosomes comes from an independent experiment in which we examined the activation of innate immune Toll-like receptors (TLRs), located within macrophage phagosomes, in response to infections with *comG*− and *comEC*− mutants. *L. monocytogenes* is known to activate TLRs when trapped in phagosomes, and this

L. monocytogenes to the cytosol even in the absence of LLO, and thus the *hly*− mutant grows intracellularly like WT bacteria in these cells (O'Riordan et al., 2002). We found that the *comG*− and *comEC*− mutants grew like the WT bacteria and *hly*− mutant in HeLa cells, indicating that *comG* and *comEC* are not required for cytosolic replication (Figure 2F). Taken together, these observations suggest that the *comG* and *comEC* genes may be involved in the process of phagosomal escape, a function that is essential in activated macrophages but dispensable in HeLa cells.

Next, the fitness of *comG*− and *comEC*− mutants during in vivo infection of mice was evaluated. C57BL/6 mice were injected intravenously with *comG*−, *comEC*−, or WT bacteria, and the bacterial counts in spleens and livers were analyzed at 72 h.p.i. As demonstrated in Figure 2G, the *comG*− and *comEC*− mutants were less able to colonize the livers and spleens of infected mice compared with WT, and a 10-fold decrease in CFUs recovered from both organs was observed. These data clearly demonstrate that the Com pseudopilus and translocation channel play important roles in *L. monocytogenes* intracellular growth and virulence.

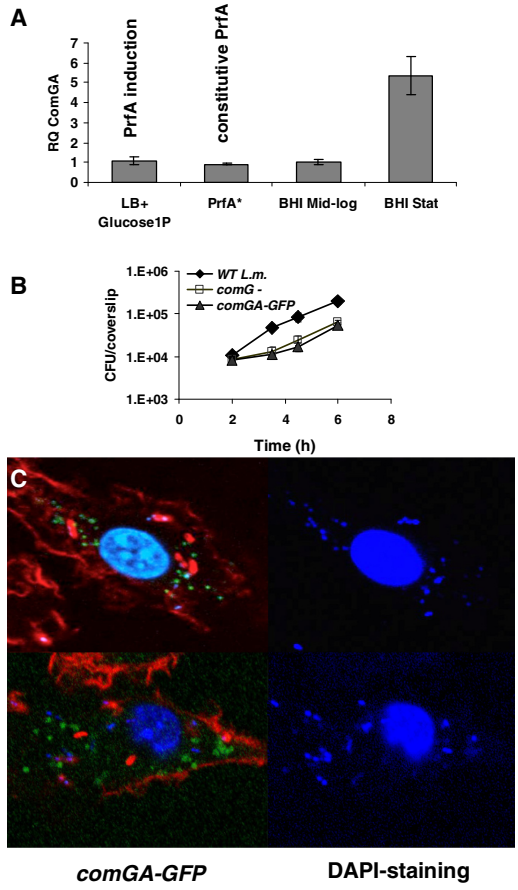

Figure 4. The Late *comG* Operon Is Expressed during the Stationary Phase and Intracellularly

(A) Analysis of *comGA* transcription levels during WT *L. monocytogenes* growth in conditions that induce PrfA activity: LB glucose-1P media during mid-exponential phase (mid-log) and in the *prfA** mutant during exponential growth in BHI medium, as well as during mid-exponential phase in BHI medium (mid-log) and stationary (stat) phase in BHI medium. Transcription levels (RQ) are relative to their levels in BHI medium during mid-exponential phase. The data represent three biological repeats. Error bars represent 95% confidence level.

(B) Intracellular growth curves of WT *L. monocytogenes* and *comG*− and *comGA-GFP* mutant strains grown in BMD macrophages. The data represent three biological repeats. Error bars represent SD.

(C) Confocal microscope images of BMD macrophages 6 h.p.i. with a *comGA-GFP* expressing strain (green). Macrophage nuclei and bacterial DNA are labeled with DAPI (blue), and actin is labeled with rhodamine phalloidin (red).

activation leads to the production of cytokines (e.g., IL-6 and IL-1β) in a manner that is dependent on the TLR adaptor protein, MyD88 (Leber et al., 2008). To examine whether *com* mutants activate an enhanced TLR-response due to their prolonged presence in the phagosomes, we measured the transcription levels of IL-6 and IL-1β upon macrophage infection. In line with our data, the *com* mutants with an intracellular growth defect (i.e., *comG*−, *comE*−, and *comEC*−) were associated with elevated transcription of IL-6 compared with WT bacteria, whereas *com* mutants that grew normally intracellularly (i.e., *comEB*−, *comEA*−, and *comFA*−) were associated with IL-6 transcript levels similar to those triggered by WT bacteria (Figure 3E). To confirm that the enhanced cytokine response is indeed triggered by TLRs in the phagosome, we repeated these experiments using MyD88−/− deficient macrophages. As expected, the enhanced induction of IL-6 and IL-1β presented by the *comG*− mutant was abolished in MyD88-deficient cells, validating that the response depends on TLRs (Figures 3F and 3G). Taken together, these results strongly support a role for the Com system in enabling *L. monocytogenes* to escape from the phagosome into the host cytosol.

To exclude the possibility that the escape defect of the *com* mutants is due to an effect on LLO, PlcA, and PlcB, we examined the transcription, secretion, and activity levels of these virulence factors in the *com* mutants in comparison with WT bacteria. First, in vitro conditions known to induce the production of virulence factors (i.e., growth in Luria Bertani [LB] glucose-1P medium to stationary phase; Ripio et al., 1997) were verified to support the induction of the Com system (Figure S2A). Under these conditions, *hly*, *plcA*, and *plcB* transcription levels in the WT bacteria, *comG*−, and *comEC*− mutants were observed to be similar (Figure S2B). The secretion and activity levels of LLO, PlcA, and PlcB proteins were also similar in the WT bacteria and *com* mutants (Figures S2C–S2G), indicating that the Com system has no effect on the known virulence factors that mediate escape.

The Majority of Intracellular *L. monocytogenes* Bacteria Express the *com* Genes

Having established that the Com apparatus is important during *L. monocytogenes* infection, we investigated how this system is regulated intracellularly. Initially, we tested the possibility that the master virulence activator of *L. monocytogenes*, PrfA, is involved. The transcription level of *comGA*, serving as a marker for the late *com* genes, was analyzed under growth conditions that activate PrfA (i.e., LB glucose-1P medium) and in the *prfA** mutant, which expresses a constitutively active PrfA protein (Miner et al., 2008). In both cases, no effect on the transcription level of *comGA* was observed, indicating that PrfA does not regulate the Com apparatus (Figure 4A). Nevertheless, we noticed that when bacteria arrived at the stationary phase, *comGA* transcription was induced (Figure 4A and Figure S2A). This phenomenon is known to occur in *B. subtilis* due to the quorum-sensing mechanism, although only 10%–20% of the bacteria express ComK at this transition (Berka et al., 2002; Maamar and Dubnau, 2005). As mentioned above, this mechanism is missing in *L. monocytogenes*, yet it remained a possibility that only a small proportion of intracellular bacteria express the *com* genes. To evaluate the percentage of *L. monocytogenes* bacteria that express the late *com* genes during intracellular growth, we adapted an experiment performed in *B. subtilis* by Hahn et al. (2005). A translational fusion of green fluorescent protein (GFP) to the carboxy-terminus of ComGA was constructed chromosomally. In *B. subtilis* it was shown that up to 20% of the bacteria are ComGA-GFP labeled under competence-inducing conditions. It was also noted that the ComGA-GFP fusion interferes with ComGA activity and pseudopilus formation, resulting in a reduced competence (Hahn et al., 2005). Similarly, we found that in *L. monocytogenes*, the ComGA-GFP fusion interfered with ComGA activity because

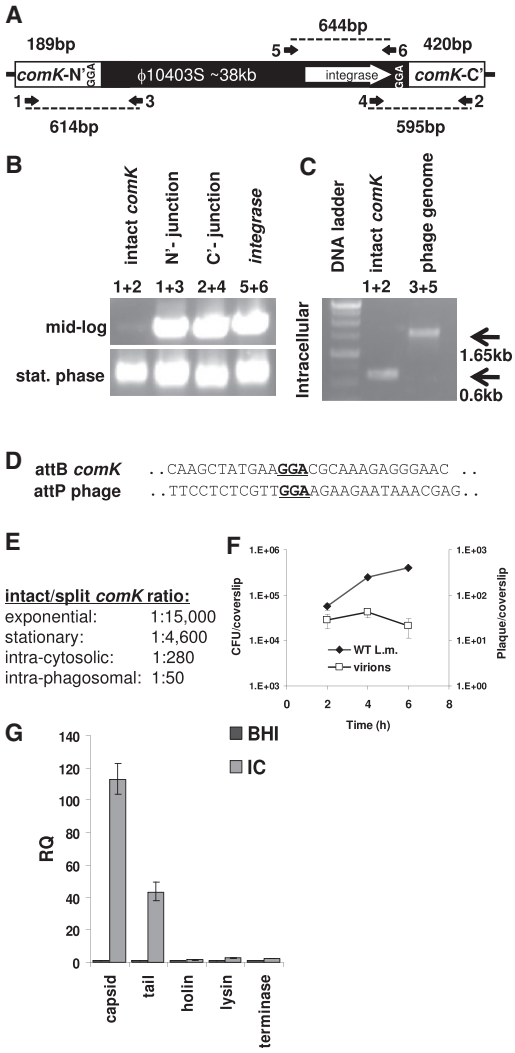

Figure 5. The Prophage Is Excised during *L. monocytogenes* Growth

(A) Schematic representation of the split *comK* gene containing the ϕ10403S prophage. Black arrows depict primers used to characterize the *comK-phage* genomic region (primers 1–6; Table S2). All PCR products were designed to be ~600 bp, including the products corresponding to the intact *comK* gene (primers 1+2) and the phage integrase gene (primers 5+6).

(B) PCR analysis of *comK-phage* genomic region in *L. monocytogenes* WT bacteria grown to exponential (mid-log) or stationary (stat) phase in BHI medium.

(C) PCR analysis of an intact *comK* gene and excised phage genome during WT *L. monocytogenes* growth in BMD macrophage cells at 6 h.p.i.

(D) DNA sequences of the *comK attB* site and the phage *attP* site in the PCR products presented in (C).

(E) The prophage excision rate was analyzed by calculating the ratio of intact/split *comK* genes in WT *L. monocytogenes* bacteria grown to exponential phase or stationary phase in BHI medium and during intracellular growth. The intracytosolic ratio represents bacteria grown intracellularly at 6 h.p.i., and the intraphagosomal ratio represents bacteria located in phagosomes at 2 h.p.i. (using the *hly*− mutant). Ratios were calculated as [intact *comK*/*16S rRNA*]/[split *comK*/*16S rRNA*] by RT-qPCR analysis.

(F) Phage plaque assay for BMD macrophages infected with WT *L. monocytogenes*. At indicated time points during infection, the number of phage PFUs was evaluated. The data represent six independent repeats. Error bars indicate the standard error.

(G) RT-qPCR analysis of the transcription levels of phage-encoded genes (capsid, tail, holin, lysin, and terminase; Table S2) during intracellular growth of WT *L. monocytogenes* in macrophage cells. Transcription levels are represented as the RQ relative to levels in BHI medium during mid-exponential phase. Error bars represent 95% confidence level.

See also Figure S3.

this strain exhibited defective intracellular growth similar to that observed for the *comG*− mutant (Figure 4B). However, in contrast to *B. subtilis*, fluorescent microscopy revealed that the majority of intracellular bacteria expressed the ComGA-GFP fusion. Accordingly, these bacteria were not associated with actin tails, consistent with a requirement for the ComG pseudopilus to escape into the cytosol (Figure 4C). Quantification revealed that ~80% of intracellular bacteria expressed the *comG* operon, which accords with the profoundly defective intracellular growth phenotype exhibited by the *L. monocytogenes comG*− mutant.

The *comK*-Associated Prophage Preferentially Excises during Intracellular Growth, Resulting in an Intact *comK* Gene

To search for transcription regulators that regulate the late *com* genes in *L. monocytogenes* during intracellular growth, we examined the promoter regions of the three *com* operons (*comG*, *comE*, and *comF*) for potential binding sites. In all of these regions, at least one pair of putative ComK-box (K-box; AAAA N_5 TTTT) was identified (Figure S3A; Hamoen et al., 1998). We determined that these motifs are highly conserved among pathogenic strains of *Listeria* and less conserved in nonpathogenic or less-pathogenic strains (Figure S3B). One of the AT-boxes (upstream of *comGA*) was completely missing in the latter. In addition, we discerned that ComK is conserved among *Listeria* species irrespective of the presence of a prophage, with very low levels of sequence divergence, implying that ComK has a functional role in *Listeria* (Figure S3C). On the basis of these observations, we surmised that ComK is a promising candidate for regulating the late *com* genes during *L. monocytogenes* infection.

To determine whether an intact and functional ComK is in fact expressed during *L. monocytogenes* infection, we monitored the chromosomal region of the *comK* gene and its integrated prophage (ϕ10403S) during intracellular growth. To that end, we designed pairs of primers to amplify both of the *comK*-prophage junctions (DNA regions overlapping with either *attBP′* or *attPB′* sites) and the prophage integrase gene, which is located immediately upstream of the *comK* 3′-truncated gene (Figure 5A; Table S2). Initially, we grew *L. monocytogenes* WT bacteria in BHI medium to mid-log or stationary phase, and then purified and PCR-amplified the bacterial genomic DNA using the different primers. We found that PCR products corresponding to the 5′-*comK*- prophage junction [N′-junction (primers 1+3)], the prophage-*comK*-3′ junction [C′-junction (primers 2+4)], and the prophage integrase gene (primers 5+6) were amplified in the presence of DNA extracted from exponentially growing bacteria (mid-log), demonstrating that indeed the prophage is integrated within the *L. monocytogenes* 10403S *comK* gene (Figure 5B). Conversely, the PCR product corresponding to an intact *comK* gene was not amplified (primers 1+2 that cross

the *attB* site in a phage-free *comK* gene). In contrast, when PCR was performed using DNA extracted from bacteria in stationary phase, a product corresponding to intact *comK* gene was observed. These results reveal that during exponential growth, most bacteria retain the prophage in their genome, but upon a shift to the stationary phase, prophage excision is induced, resulting in a mixed population of bacteria (some with a disrupted *comK* gene and others with an intact one). Next, we examined the region around the *comK* gene in bacteria grown intracellularly in macrophages for 6 hr and discovered that intracellular bacteria contain an intact *comK* gene (Figure 5C). Moreover, we detected an excised form of the phage genome (possibly circular) using primers 3 and 5, which cross the phage *attP* site and generate an ~1.6 kbp fragment containing the phage genome integration site. To confirm more precisely the nature of these PCR products generated from intracellular bacteria, we sequened the intact *comK* and phage genome PCR products. Indeed, we determined that a precise excision of the phage genome leaves an in-frame coding sequence of the *comK* gene containing the GGA *attB* site, and in parallel the phage attachment GGA *attP* site is reconstituted (Figure 5D).

Next, we performed an RT-qPCR analysis to determine the ratio of intact/split *comK* genes, a measure that is representative of phage excision rates. We assessed the level of intact *comK* genes using specific primers that amplify the *attB* site region, and the level of split *comK* genes using primers that recognize the *comK* N′-junction (Table S2). First, this ratio was determined for bacteria grown to mid-log versus stationary phase in BHI media, and indeed a higher rate of prophage excision was detected during the stationary phase (1:4,600 versus 1:15,000 in exponential phase; Figure 5E). We then measured this ratio in intracellular bacteria 6 h.p.i. and in phagosomally trapped bacteria 2 h.p.i. (using the *hly*− mutant). Remarkably, we found that phage excision was highly induced during intracellular growth (1:280), particularly when the bacteria were trapped within phagosomes (1:50; Figure 5E), which suggests that prophage excision is triggered within the phagosome compartment. This observation is consistent with the requirement for the Com system during phagosomal escape and indicates specific regulation of phage excision during *L. monocytogenes* infection.

Upon switching from lysogeny to the lytic pathway, phage excision normally leads to generation of progeny virions and bacterial lysis. Therefore, we tested the possibility that the prophage turns into a lytic phage during *L. monocytogenes* intracellular growth. We searched for infective virions in *L. monocytogenes*–infected macrophages using a phage plaque assay (Hodgson, 2000). Only a residual number of plaque-forming units (PFUs; n ~ 50) were detected in lysates of *L. monocytogenes*–infected macrophages, and this number was steady throughout the course of infection (Figure 5F). This result indicates that propagation and release of virions does not occur during *L. monocytogenes* intracellular growth, and suggests that this process is somehow prevented. To corroborate this premise, we measured the transcription levels of several phage-encoded genes during *L. monocytogenes* intracellular growth. In line with a lack of bacterial lysis inside macrophages, we found that, whereas the structural genes encoding capsid and tail proteins were highly induced intracellularly, the phage genes responsible for bacterial lysis (i.e., phage holin and lysin), as well as the phage terminase gene, which is responsible for DNA packaging, were all uninduced (Figure 5G). Overall, these results suggest that phage propagation is blocked during *L. monocytogenes* infection of macrophage cells.

ComK Regulates the Late *com* Genes and Is Required to Promote Intracellular Growth

Given all the data pointing to ComK as the regulator of the *com* genes during intracellular growth, we examined whether a functional ComK protein is required for *L. monocytogenes* intracellular growth. To that end, we generated several mutant strains. First, strains were constructed that bear deletions of the *comK* 5′-terminal part (*comKN*− mutant), the *comK* 3′-terminal part (*comKC*− mutant), or the phage integrase gene (*int*− mutant), the latter of which is suspected to be responsible for prophage excision. Of note, all deletion mutants grew like WT bacteria in BHI broth (Figure S1A). First, we observed that an intact *comK* gene was not detectable in the *int*− mutant, establishing that the product of this gene mediates prophage excision (Figure 6A). Intracellular growth curves revealed that *comKN*− and *comKC*− mutants were defective in intracellular growth in macrophage cells, a phenotype that was complemented by introducing an intact *comK* gene under the regulation of its native promoter (pPL2-*comK*; Figure 6B). These experiments validated that the *comK* gene is indeed necessary for efficient intracellular growth. Of note, the intracellular growth curve of the *int*− mutant also indicated a growth defect, which was complemented by introducing the *int* gene itself (pPL2-*int*; Figure 6C). As expected, the growth defect of the *int*− mutant was complemented effectively by introducing a complete (i.e., intact) *comK* gene (pPL2-*comK*), indicating that phage excision is necessary specifically for the formation of an intact *comK* gene (Figure 6C).

In a reciprocal experiment, the intracellular growth ability of a phage-cured strain (containing an intact *comK* gene and termed *cured L.m.*; Lauer et al., 2002) was compared with an isogenic mutant deleted of the *comK* gene (*cured comK*− mutant; Table S1) and WT bacteria. As shown in Figure 6D, although the *cured comK*− mutant grew normally in BHI broth (Figure S1A), it exhibited defective intracellular growth in macrophage cells. Introducing the pPL2-*comK* plasmid to the *cured comK*− mutant complemented this intracellular growth defect, strengthening the premise that ComK is produced during infection and that its function is required.

In the light of our data indicating that phage excision is necessary for efficient intracellular growth, we suspected that other A118-like prophages might play a similar role. However, *Listeria* A118-like prophages exhibit extremely high genomic diversity (Dorscht et al., 2009). For example, the phage of another key *L. monocytogenes* laboratory strain, EGDe, is highly divergent from the ϕ10403S-phage, exhibiting only 42 similar genes out of a total of 70 predicted genes. To explore whether the EGDe phage can support efficient excision, we introduced *L. monocytogenes* EGDe phage (ϕEGDe) to the 10403S *L.m. cured* strain and analyzed intracellular growth. *L. monocytogenes* 10403S strains harboring either the native phage (ϕ10403S) or the ϕEGDe-phage grew similarly in

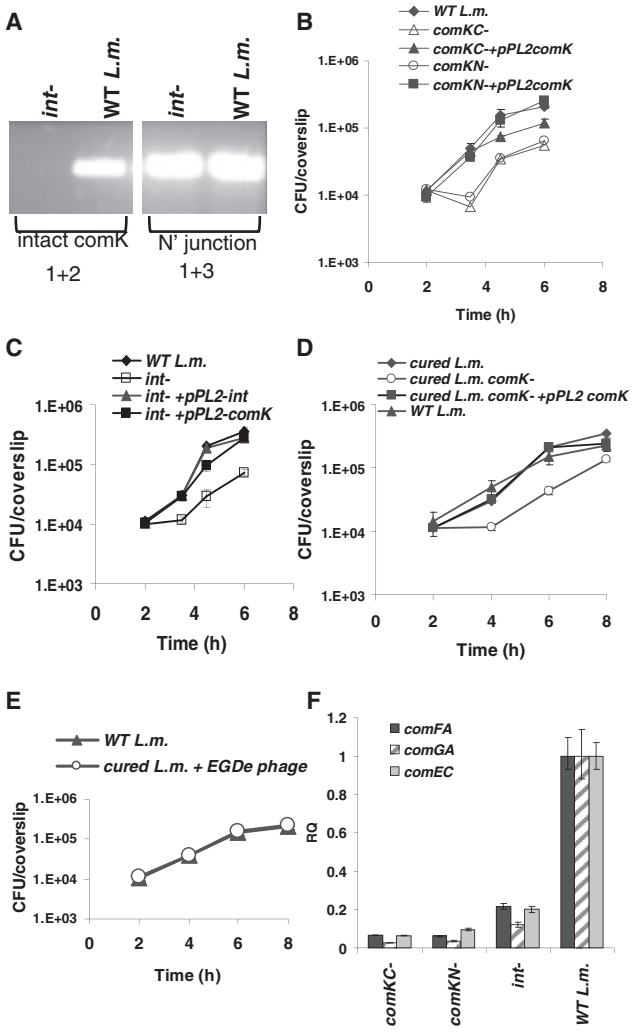

Figure 6. ComK Is Required for Intracellular Growth and for Activation of the Late *com* Genes

(A) PCR analysis of an intact *comK* gene and *comK*-phage N′ junction in WT *L. monocytogenes* and in the *int*− (integrase) mutant during growth in stationary phase in BHI medium.

(B) Intracellular growth curves of WT *L. monocytogenes* and *comKN*− and *comKC*− mutants grown in BMD macrophage cells, as well as mutants complemented with an intact copy of *comK* gene (pPL2-*comK*).

(C) Intracellular growth curves of WT *L. monocytogenes*, *int*− mutant, and *int*− mutant complemented with an intact copy of the *int* gene (pPL2-*int*) and the *comK* gene (pPL2-*comK*), grown in BMD macrophage cells.

(D) Intracellular growth curves of WT *L. monocytogenes*, a phage-cured *L. monocytogenes* strain (*cured L.m.*), and its isogenic mutant with the *comK* gene deleted (*cured L.m. comK*−), as well as a complemented strain with an intact *comK* gene (pPL2-*comK*), grown in BMD macrophage cells.

(E) Intracellular growth curves of the WT *L. monocytogenes* 10403S strain harboring its native prophage (ϕ10403S) in comparison with the 10403S strain harboring ϕEGDe-prophage in BMD macrophage cells. In all growth curves, the data represent three independent repeats. Error bars represent SD.

(F) RT-qPCR analysis of transcription levels of late *com* genes (*comFA*, *comGA*, and *comEC*) in WT *L. monocytogenes* bacteria and *comKC*−, *comKN*−, and *int*− mutants during stationary phase. Transcription levels are represented as the RQ relative to their levels in WT *L. monocytogenes*. Error bars represent 95% confidence level.

See also Figure S1A and Table S1.

macrophage cells, indicating that both phages initiate efficient excision during infection (Figure 6E).

Finally, to corroborate the notion that ComK regulates the expression of the late *com* genes, we analyzed the transcription levels of representative genes (*comGA*, *comEC* and *comFA*) in WT bacteria as well as in *comKN*−, *comKC*−, and *int*− mutants during the stationary phase. As expected, all three late *com* genes were induced in WT bacteria under these conditions (Figure 6F). However, this induction was dependent on the formation of an intact *comK* gene because it was not observed in any of the three mutant strains. Taken together, our findings establish that the *L. monocytogenes* ComK protein is a transcriptional activator of the late *com* genes.

DISCUSSION

In this study we characterized the genome rearrangement and virion production of the ϕ10403S-prophage, and evaluated the impact of its integration within *comK* on the virulence of *L. monocytogenes*. Although this family of *Listeria* prophages is known to be infective and capable of transduction (Hodgson, 2000; Loessner et al., 1995), here we show a unique adaptation of the prophage to the intracellular lifestyle of its bacterial host. Overall, the data support a model in which the phage excises its genome during bacterial phagocytosis, yielding an intact *comK* gene that produces a functional ComK protein. ComK in turn is needed to activate the expression of the Com system to allow efficient phagosomal escape. This excision event appears to be highly efficient, as bacteria with or without the phage grew the same intracellularly, whereas bacteria that retained the phage (*int*− mutant) were defective in intracellular growth. Of note, we demonstrated that this excision event is not unique to ϕ10403S-prophage but also occurs efficiently in the case of another prophage from a different *L. monocytogenes* strain.

A critical step in the regulatory mechanism identified in this study is the precise excision of ϕ10403S-prophage from the *L. monocytogenes* genome. This step was shown to require the phage integrase gene; however, the upstream events that lead to activation of the integrase gene remain unclear. Nevertheless, it is notable that phage excision was induced primarily when bacteria were located in phagosomes, which raises the possibility that conditions within this compartment signal for phage excision. Generally, prophages excise their genomes and produce virions when their hosts are subjected to stress conditions such as oxidative stress, DNA damage, and nutritional changes (Johnson et al., 1981; Little and Mount, 1982), conditions that are characteristic of phagosomes. Remarkably, induction of ϕ10403S-prophage excision did not lead to propagation and release of progeny virions, a process that is accompanied by bacterial lysis, which suggests that virion production is actively aborted during *L. monocytogenes* intracellular growth. In the light of these findings, we surmise that bacterial lysis within the cytosolic niche is not beneficial for the phage (or for the bacteria), because the phage is not adapted to survive within mammalian cells. One interpretation of our data is that the phage possesses a previously unidentified regulatory mechanism (different from the well-characterized lysogenic and lytic mechanisms) that controls propagation in such a way that genome

excision is not coupled to virion production and bacterial lysis. Alternatively, it is possible that *L. monocytogenes* itself somehow regulates this phage mechanism and induces *comK* production while preventing lysis. In summary, our study reveals a distinct mode of regulation that turns ϕ10403S-prophage into a genetic switch that plays a biologically important role in the intracellular lifecycle of *L. monocytogenes*.

Prophages are known to contribute to the pathogenicity of their bacterial hosts primarily via the production of phage-encoded virulence factors (Brüssow et al., 2004). In contrast, there are only a few instances in which genomic rearrangement of prophage DNA was shown to influence bacterial gene expression (Kirby et al., 1994; Kunkel et al., 1990; Scott et al., 2008). A well-characterized example is the regulation of sigma K (σ^K) factor during sporulation in *B. subtilis.* Similarly to *comK*, the *sigK* gene is split by an ~42 kb DNA element that excises only in the mother cell, resulting in expression of σ^K mother cell specific genes (Kroos et al., 1989; Kunkel et al., 1990; Stragier et al., 1989). Another classic (though not phage-related) example of gene regulation by an interrupting DNA element is that which occurs in cyanobacteria during developmental differentiation of heterocysts (Golden et al., 1985). Although these examples demonstrate exploitation of phage genomes as regulatory elements, they all involve cryptic prophages or remnants that have lost the ability to produce infective phage particles. In contrast to these examples, the work presented here demonstrates the regulation of bacterial genes/virulence by an active (infective) phage.

Although *L. monocytogenes* is missing most of the regulatory machinery of the Com system, we found some fundamental similarities between the *com* genes expression patterns of *L. monocytogenes* and *B. subtilis*, namely, in both bacteria, the late *com* genes are induced upon entry to the stationary phase and both require ComK for their activation. Whereas in *B. subtilis*, ComK is regulated at the level of protein stability, in *L. monocytogenes* it is the integrity of the *comK* gene that is controlled. Specifically, in *B. subtilis*, ComK is constitutively expressed and targeted to degradation via binding to the MecA protein. Of note, although the genome of *L. monocytogenes* encodes a MecA homolog, it has not been reported to regulate ComK; rather, it was implicated in the regulation of another virulence factor, SvpA, which is also important for phagosomal escape (Borezee et al., 2000, 2001).

Our study uncovered an unexpected function for the major components of the Com system during intracellular growth of *L. monocytogenes.* We found that the Com pseudopilus and membrane translocation channel are necessary for efficient phagosomal escape, as well as for virulence in mice, whereas DNA binding components are dispensable. We suspect that ComEC and ComG cooperate with LLO during phagosomal escape because they all are dispensable for growth in HeLa cells yet critical for phagosomal escape in activated macrophage cells. This proposed function correlates directly with the enhanced excision rate of the phage within phagosomes and the high expression of the *com* genes during infection.

Lastly, because the Com system resembles the type IV and type II secretion systems (Chen and Dubnau, 2004; Fronzes et al., 2009), it is possible that *L. monocytogenes* exploits core components of the Com system to export proteins to promote phagosomal escape. Another possibility is that the formation of pseudopilus on the bacterial surface exerts a physical force against the phagosomal membrane, thereby easing its perforation. Further studies are required to explore these possibilities. Overall, this study highlights the complexity of host-pathogen-phage interactions, and provides evidence that a bacteriophage modulates the virulence of its bacterial host in the course of a mammalian infection.

EXPERIMENTAL PROCEDURES

Strains, Cells, and Growth Conditions

L. monocytogenes 10403S strain was used as the WT bacteria and as the parental strain to all in-frame deletions generated in this work. The bacterial strains used in this study are listed in Table S1. The generation of mutants is described in Extended Experimental Procedures. For infection experiments, *L. monocytogenes* bacteria were grown overnight in BHI at 30°C without agitation. For induction of PrfA activity, LB MOPS glucose 1P medium was used (Ripio et al., 1997). BMD macrophages were isolated from C57BL/6 female mice (6–8 weeks old; Harlan Laboratories, Jerusalem, Israel) and cultured as described previously (Portnoy et al., 1988). G. Nussbaum (Hebrew University of Jerusalem, Jerusalem, Israel) supplied the MyD88−/− deficient BMD macrophage cells, which were originally a gift from Dr. S. Akira (Osaka University, Osaka, Japan). For macrophage activation, 1 ng/ml of recombinant murine interferon-γ (IFNγ; PeproTech) was added for 36 hr before and during infection. Intracellular growth analysis was performed as described previously (Herskovits et al., 2007). Briefly, BMD macrophages grown on glass coverslips were infected with *L. monocytogenes* bacteria at a multiplicity of infection (MOI) of one. Thirty minutes postinfection, the macrophage cells were washed, and gentamicin (5 μg/ml) was added at 1 h.p.i. At each time point, three coverslips were taken for numeration of intercellular bacteria (further details are provided in Extended Experimental Procedures). Phagosomal escape assay was performed as described previously (Glomski et al., 2002). For analysis of intracellular *L. monocytogenes* gene expression, 25×10^6 BMD macrophage cells on a 150 mm Petri dish were infected with 1×10^8 bacteria. At 6 h.p.i., the macrophages were lysed in 20 ml of ice-cold water and then the released bacteria were collected on 0.45 μm HA filters (catalog No. HAWP04700; Millipore). Bacterial RNA purification, gene expression analysis, and microarray analysis are described in Extended Experimental Procedures. Primers are listed in Table S2. For mice infections, C57BL/6 female mice (6–8 weeks old; Harlan Laboratories) were infected via tail vein injections with 4×10^4 bacteria. Spleens and livers were harvested at 72 h.p.i. and homogenized in 0.2% saponin. The bacterial viable count in each organ was determined by plating of homogenates onto agar plates.

Plaque Assay, Phage Induction, and Infection

For the plaque assay, we used the *L. monocytogenes* Mack861 strain. Lysates of infected macrophages were filtered through 0.45 μm filters and spread on a lawn of the Mack861 strain plate. After 4 days of incubation, the PFUs were counted. Phage induction from *L. monocytogenes* EGDe lysogen was achieved by UV irradiation, and free bacteriophages were used to infect *L. monocytogenes* 10403S-cured strain. A more detailed description is provided in Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.06.036.

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